Eur. J. Clin. Chem. Clin. Biochem.
Vol. 29, 1991, pp. 385–389

Determination of Pyrimidine Deoxynucleoside Triphosphates in Leukaemia Cell Extracts Containing 1-β-*D*-Arabinofuranosylcytosine Triphosphate

By *P. Chiba*[1], *T. Szekeres*[1] and *W. Jäger*[2]

[1] *Department of Medical Chemistry*
[2] *Department of Pharmaceutical Chemistry*
University of Vienna, Austria

(Received July 24, 1990/March 12, 1991)

Dedicated to Professor Dr. Erich Kaiser on the occasion of his 65th birthday

Summary: Deoxynucleoside 5′-triphosphates (dNTPs) can be determined in cell extracts by high performance liquid chromatography after prior selective degradation of ribonucleoside 5′-triphosphates with sodium periodate and methylamine. When the method is used for the evaluation of deoxynucleoside triphosphates in 1-β-*D*-arabinofuranosylcytosine triphosphate (ara-CTP)-containing cell extracts, an additional peak coeluting with thymidine triphosphate (dTTP) is observed. This peak is due to the formation of a carboxylic acid derivative of ara-CTP by periodate oxidation, and it can lead to considerable overestimation of dTTP. Formation of this peak can be avoided by using alkaline reaction conditions (pH 7.5) and by changing the sequence of addition of the reagents used in the periodation procedure. By employing this modified protocol, cellular dNTP and ara-CTP levels can be monitored in extracts of leukaemic blasts during cytosine arabinoside treatment in two separate HPLC runs.

Introduction

The measurement of deoxyribonucleoside-5′-triphosphates (dNTPs) has gained widespread importance in the study of anticancer and antiviral agents. An indirect assay using the respective endogenous deoxynucleoside as a limiting factor in a DNA-polymerase catalysed reaction has been described (1–3). This method cannot be used, however, when the chemotherapeutic agents themselves or their metabolites interfere with the polymerase reaction. An alternative method uses high performance liquid chromatography to separate the deoxyribonucleotides (4, 5). Since this method does not separate ribonucleotides (rNTPs) from deoxyribonucleotides, it is necessary to first chemically degrade the rNTPs, using periodate in the presence of methylamine to cleave the 2′–3′ carbon bond of ribose; the resulting dialdehyde derivative reacts with the methylamine to form a *Schiff* base. Subsequent β-elimination leads to removal of the phosphate groups from the molecule and cleavage of the N-glycosidic bond (6). Ribonucleotides are thus converted to the respective bases, which elute with the void volume of the anion exchange column.

Pyrimidine dNTP levels have been shown to influence the sensitivity of cells towards 1-β-*D*-arabinofuranosylcytosine (ara-C) (7–9). The determination of the biologically active metabolite, 1-β-*D*-arabinofuranosylcytosine triphosphate (ara-CTP), and of cellular dNTP levels in extracts prepared from blasts of the same patient is, therefore, important, when studying the mutual interference of drug and dNTP levels. Although published high performance liquid chromatography methods yield highly accurate and reproducible results for the determination of dNTPs in cell extracts, samples containing ara-CTP cannot be an-

alysed, because a derivative peak coeluting with thymidine triphosphate is formed from ara-CTP during periodation. This paper presents a modification of the method that avoids formation of this peak.

Materials and Methods

Deoxy- and ribonucleoside triphosphates, 1-β-*D*-arabinofuranosylcytosine triphosphate, sodium periodate, methylamine hydrochloride, 1,1,2-trichlorotrifluoroethane (freon), tri-*n*-octylamine and rhamnose were from Sigma Chem. Comp. (St. Louis, MO, USA).

Cell culture and preparation of cell extracts

The KG1 myoblast cell line (10) was cultured in RPMI 1640 medium (fetal calf serum, volume fraction 0.1, 10 mg/l gentamycin) in a humidified atmosphere containing 5% CO_2. After chilling on ice, 5×10^7 cells were centrifuged at 500 *g*, the cell pellet was resuspended in 100 µl phosphate-buffered saline containing 111 kBq of [8-^{3}H]adenine (666 GBq/mol, 37 GBq/l, Amersham Int., Amersham, UK; to allow correction for cell pellet volume and dilution during periodation) and 6 µl of 100 g/l trichloroacetic acid were added. The extract was allowed to stand on ice for 30 min and protein was sedimented by centrifugation in an Eppendorf microfuge. The supernatant was neutralized with 1.1 vol of freon containing 0.5 mol/l tri-*n*-octylamine and the protein pellet was redissolved in 1 ml of 0.5 mol/l NaOH at 37 °C for 2 h. Protein was determined according to the method of *Bradford* (11). The values obtained for dNTPs and rNTPs were normalized to protein (cell number) and to radioactivity in the sample (sample volume), which was determined prior to injection into the HPLC system by liquid scintillation counting of a 5 µl aliquot.

Periodation of ribonucleoside triphosphates

Original method

Periodation was performed according to *Garrett & Santi* (4). Briefly, to 100 µl of a neutralized cell extract or nucleotide solution was added 4 µl of 0.5 mol/l $NaIO_4$ (final concentration 20 mmol/l) followed by 5 µl of 4 mol/l methylamine (pH 7.5). After mixing, samples were incubated at 37 °C for 30 min. Residual periodate was destroyed by adding 2 µl of 1 mol/l rhamnose. Samples were put on ice and analysed as described below.

Modified method

In the modified protocol, 30 µl of 4 mol/l methylamine (pH 7.5) were added to 100 µl of neutralized cell extract followed by the addition of 8 µl of sodium periodate (0.5 mol/l). The mixture was incubated at 37 °C for 30 min after which time 2 µl of 1 mol/l rhamnose were added to remove residual sodium periodate. Samples were processed as described below.

HPLC separation of dNTPs and ara-CTP

Aliquots (100 µl) of the samples were analysed using a Varian 7500 liquid chromatograph, a Shimadzu R1-2A integrator and a 4.6 x 250 mm Partisil 10 SAX column (Whatman Ltd, Kent, UK). Samples were eluted with a 3.2 mol/l ammonium phosphate buffer, pH 3.3 (pH adjusted by addition of 3.2 mol/l H_3PO_4), containing 20 mol/l acetonitrile. Separation was performed at ambient temperature and a flow rate of 2 ml/min. The column eluate was monitored at 280 nm and 254 nm, the latter wavelength giving a stronger response for dGTP. With this modification, an isocratic separation of the 4 deoxynucleoside triphosphates and of ara-CTP was possible. The pH of the elution buffer determined the retention time of dTTP relative to that of the other 4 triphosphates, an increase in pH leading to a decrease in dTTP retention time and vice versa. Quantitation of the peaks was linear between 5 pmol and 5 nmol and with cell numbers ranging from 1×10^7 to 1×10^8.

For the experiments monitoring the breakdown of ara-CTP during periodation of ribonucleotides, 1 nmol of ara-CTP standard was added to neutralized extracts prepared from 5×10^7 KG1 cells, and samples were subjected to periodation by the original or the modified method described above.

Identification of the ara-CTP breakdown product

Peaks corresponding to the breakdown product and to ara-CTP were collected, lyophilized and reconstituted in 1 ml dimethylsulphoxide containing 1 mg 4-bromomethyl-7-methoxycoumarin and 30 mg Na_2CO_3, essentially as described by *Dunges & Seiler* (12). Samples were mixed, centrifuged, to remove undissolved sodium carbonate, and the supernatant was incubated at 40 °C for 24 h. Separation of the adducts was performed with a liquid chromatography system consisting of a Kontron 420 pump, an SFM 25 fluorimeter (Kontron, Vienna, Austria) and a Lichrosorb RP18 column (5 µm, 4.2 × 125 mm, Merck, Darmstadt, FRG). A 20 µl aliquiot was injected into the HPLC system. Substances were eluted at a flow rate of 1 ml/min with methanol/water (1+1, by vol.). Column temperature was 30 °C. The excitation wavelength was 325 nm and emission was monitored at 395 nm. The derivatized breakdown product of ara-CTP eluted with a retention time of 7.9 min and was well separated from unreacted 4-bromomethyl-7-methoxycoumarin (retention time 9.6 min). In the ara-CTP-containing sample, a 4-bromomethyl-7-methoxycoumarin-derivative peak was absent.

Results and Discussion

Degradation of ara-CTP during periodation

An incomplete, time-dependent breakdown of the 2′—3′ *trans* diol ara-CTP was observed during periodation by the method of *Garrett & Santi* (fig. 1). A change of pH from alkaline reaction conditions (pH 7.5) to slightly acidic reaction conditions (pH 5.6) had no influence on the degradation velocity.

Since breakdown of ara-CTP occurred, the accurate determination of ara-CTP was only possible in samples that had not been subjected to periodate treatment. HPLC conditions had to be modified to allow resolution of the ara-CTP peak. An isocratic elution using 3.2 mol/l ammonium phosphate buffer (pH 3.3) containing 20 mol/l acetonitrile allowed separation of ara-CTP (retention time 14.5 min) from CTP (retention time 12.0 min) and the ATP/UTP peak (17.4). A separation profile is shown in figure 2.

During periodation, ara-CTP was mainly converted to the corresponding base, cytosine, which eluted with a retention time of 2.6 min. In addition, a peak with a retention time of 17.0 min was observed. This peak was more readily formed at pH 6.5 (fig. 3a) but could

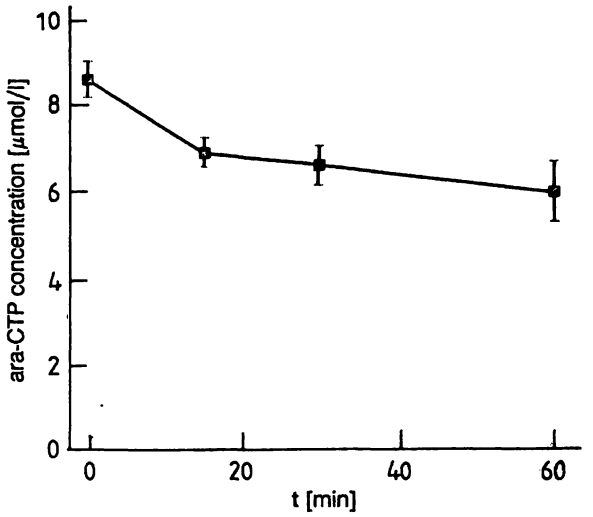

Fig. 1. Breakdown of ara-CTP in cell free extracts as a function of periodation time. 5×10^7 KG1 cells were acid extracted after prior treatment with 100 µmol/l ara-C and periodation was performed according to the method of *Garrett & Santi* (4) for the time indicated. Data points represent the mean ± SD of 4 determinations.

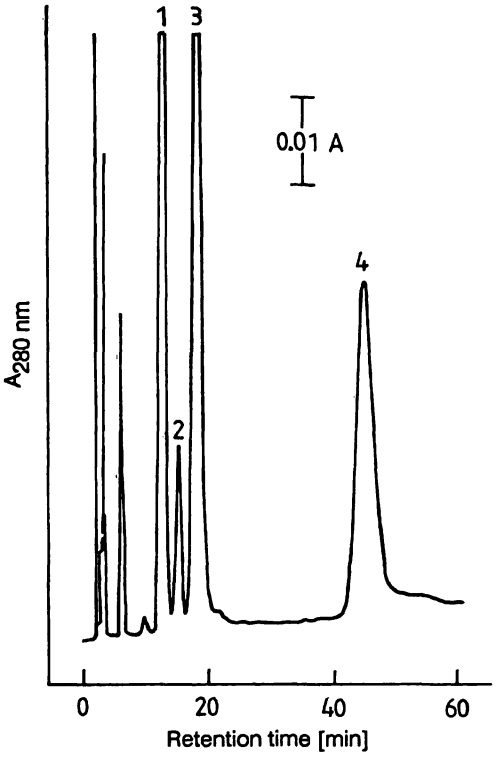

Fig. 2. Detection of ara-CTP in acid extracts of KG1 cells. 5×10^7 cells were treated with 100 µmol/l ara-C for 60 min prior to acid extraction. Neutralized samples were analysed directly. Peaks 1, 2, 3 and 4 correspond to CTP, ara-CTP, ATP/UTP and GTP, respectively.

also be detected when periodation was performed at pH 7.5 (fig. 3b). The derivative peak represented 1.3 and 0.3% of total UV absorbing material at pH 6.5 and 7.5, respectively. When analysing cell extracts, coelution of this peak with thymidine triphosphate was observed.

Analysis of the ara-CTP breakdown product

Peaks corresponding to ara-CTP and to the breakdown product were collected and derivatized with 4-bromomethyl-7-methoxycoumarin. The reaction mixture was then separated by high performance liquid chromatography as detailed in the Methods section. A reaction product with increased hydrophilicity was detected when the derivative peak, but not the authentic ara-CTP peak, was reacted. This was indicative of the presence of a molecule containing a carboxyl group.

In addition, the increase in retention time from 14.5 min (ara-CTP) to 17 min and the favoured formation at acidic pH also suggest that the arabinose moiety was oxidized to a carboxylic acid derivative during periodation.

Prevention of the formation of the ara-CTP breakdown product

Periodation conditions were modified to avoid formation of the peak coeluting with thymidine triphosphate. This proved necessary since levels of the ara-C triphosphate in leukaemic blasts have been reported to be at least an order of magnitude higher than

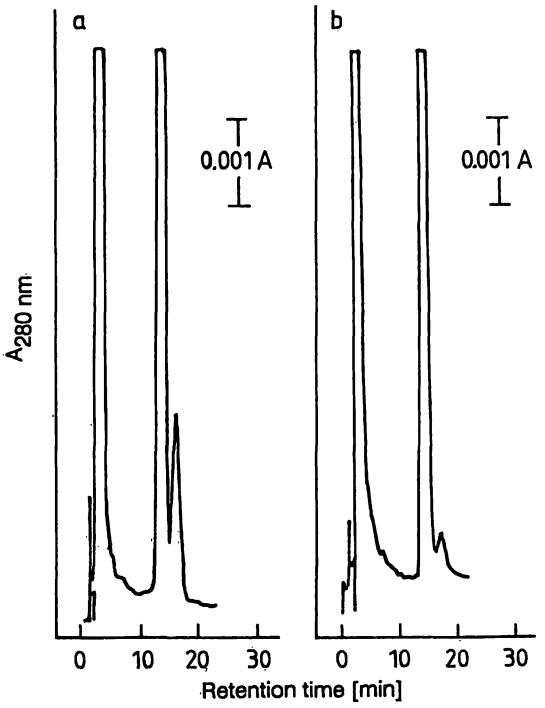

Fig. 3. Formation of the degradation product coeluting with dTTP. 1 nmol of ara-CTP standard was subjected to periodation at pH 6.5 (a) and 7.5 (b). The periodation time was 30 min. The peak area of the derivative peak corresponded to 1.3 and 0.3% of total UV-absorbing material, respectively.

dTTP levels (13–15), and even a small amount of degradation product was likely to lead to considerable overestimation fo the dTTP peak. Since the derivative peak appeared much smaller when oxidation was performed under alkaline reaction conditions, a pH of 7.5 was selected. Periodate-mediated conversion of the ribose moiety to the dialdehyde derivative has been reported to proceed to completion within minutes (6). In the case of arabinose, the dialdehyde intermediate could, at least partially, undergo further oxidation to the carboxylic acid derivative in the absence of methylamine. Formation of this product could be assumed to be minimal if methylamine was added prior to periodate, since an immediate coupling of the dialdehyde derivative to a *Schiff* base would be favoured. Table 1 gives a comparison of dCTP and dTTP levels in ara-CTP-containing samples, which were periodated by the method of *Garrett & Santi* or by our modified protocol. For this experiment, KG1 cell extracts were prepared and ara-CTP standard was added to neutralized samples before periodation. The ara-CTP concentration was chosen to be comparable to intracellular levels achieved in leukaemic blasts during ara-C treatment in vivo (13, 14). dTTP levels were shown to be identical in controls which had been subjected to periodate oxidation in the absence of ara-CTP, and in ara-CTP containing samples, which had been treated according to the modified protocol. In contrast, a 66% overestimation of the dTTP pool was observed when using the original method. A representative separation profile obtained with the modified protocol is shown in figure 4. Peaks 1–5 correspond to dCTP, ara-CTP, dTTP, dATP and dGTP, respectively. Table 2 shows that under these modified reaction conditions recovery of the respective dNTPs was comparable to that reported in the literature (4, 5), and that degradation of ribonucleoside triphosphates was complete. Ara-CTP was converted only to the corresponding base, cytosine. No additional UV absorbing peaks were observed. This modified protocol allows quantitation of all four deoxynucleoside triphosphates in ara-CTP-containing samples. It therefore enables the study of the mutual interference of pyrimidine deoxynucleoside triphosphate levels with ara-C incorporation in cell lines and in blasts of leukaemia patients during ara-C therapy.

Tab. 1. Periodation of KG-1 cell extracts with and without added ara-CTP

	ara-CTP	dCTP	dTTP	dCTP/dTTP
Original method (4)	–	1.54 ± 0.16	3.38 ± 0.34	0.46
	+	1.61 ± 0.07	5.61 ± 0.71	0.29
Modified method	+	1.67 ± 0.18	3.32 ± 0.36	0.50

1 nmol of ara-CTP standard was added to cell extracts prepared from 5×10^7 KG1 cells prior to periodation. The dCTP and dTTP levels were compared with those found in extracts that had been periodated in the absence of ara-CTP. Data are given as pmol/10^6 cells and represent the mean ± SD of triplicate determinations. See the Methods section for details.

Tab. 2. Recovery of deoxynucleoside triphosphate standards after periodation according to the modified protocol

	dNTPs	dNTPs + rNTPs
dCTP	99.3 ± 0.4	98.5 ± 1.6
dTTP	100.0 ± 2.9	94.0 ± 1.7
dATP	99.9 ± 0.6	98.0 ± 2.2
dGTP	96.9 ± 4.1	89.0 ± 6.9

dNTP standards (20 µmol/l) or a mixture of dNTP standards (20 µmol/l) and rNTP standards (1 mmol/l) were subjected to periodation according to the modified protocol. Recoveries are given as percentages of sham treated controls and represent the mean ± SD of triplicates.

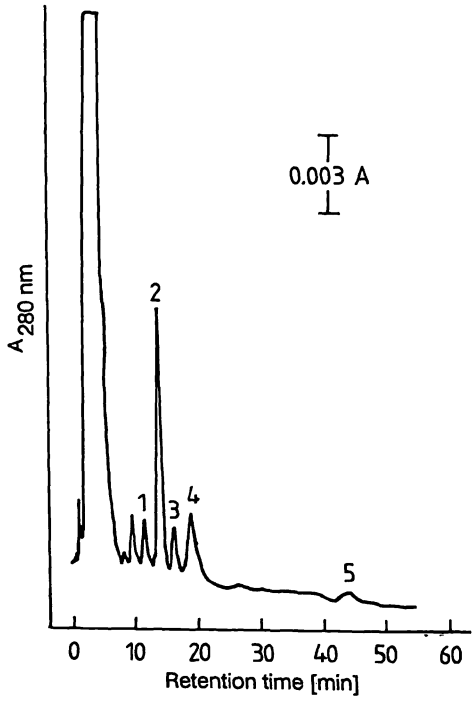

Fig. 4. Separation profile of dNTPs in extracts prepeared from 5×10^7 KG1 cells after treatment of whole cells with 100 µmol/l ara-C for 60 min. Peaks are dCTP (1), ara-CTP (2), dTTP (3), dATP (4) and dGTP (5). Since degradation of ara-CTP occurred during periodation of ribonucleotides, this triphosphate could only be determined in extracts which had not been treated with sodium periodate (cf. fig. 2). Peaks 1–4 were detected at 280 nm to increase the absorption of the pyrimidine deoxynucleotides, and the dGTP peak was detected at 254 nm.

Acknowledgement

This work was supported by grants from "Medizinisch-Wissenschaftlicher Fonds des Bürgermeisters der Bundeshauptstadt Wien", "Kommission Onkologie der Medizinischen Fakultät der Universität Wien" and "Anton Dreher-Gedächtnisschenkung für Medizinische Forschung".

References

1. Solter, A. W. & Handschumacher, R. E. (1969) A rapid quantitative determination of deoxyribonucleoside triphosphates based on the enzymatic synthesis of DNA. Biochim. Biophys. Acta *174*, 585–590.
2. Skoog, L. (1970) An enzymatic method for the determination of dCTP and dGTP in picomol amounts. Eur. J. Biochem. *17*, 202–208.
3. Lindberg, U. & Skoog, L. (1970) A method for the determination of dATP and dTTP in picomol amounts. Anal. Biochem. *34*, 152–160.
4. Garrett, C. & Santi, D. V. (1979) A rapid and sensitive high pressure liquid chromatography assay for deoxynucleoside triphosphates in cell extracts. Anal. Biochem. *99*, 268–273.
5. Tanaka, K., Yoshioka, A., Tanaka, S. & Wataya, Y. (1984) An improved method for the quantitative determination of deoxyribonucleoside triphosphates in cell extracts. Anal. Biochem. *139*, 35–41.
6. Neu, H. C. & Heppel, L. A. (1964) Nucleotide sequence analysis of polyribonucleotides by means of periodate oxidation followed by cleavage with an amine. J. Biol. Chem. *239*, 2927–2934.
7. Robert de Saint Vincent, H. C. & Buttin, G. (1980) Studies on 1-β-D-arabinofuranosylcytosine resistant mutants of chinese hamster fibroblasts. IV. Altered regulation of CTP synthetase generates arabinosylcytosine and thymidine resistance. Biochim. Biophys. Acta *610*, 352–359.
8. Weinberg, G., Ullman, B. & Martin, D. W. (1981) Mutator phenotypes in mammalian cell mutants with distinct biochemical defects and abnormal deoxyribonucleoside triphosphate pools. Proc. Natl. Acad. Sci. USA *78*, 2447–2451.
9. Bianchi, V., Pontis, E. & Reichard, P. (1987) Regulation of pyrimidine deoxyribonucleotide metabolism by substrate cycles in dCMP deaminase-deficient V79 hamster cells. Mol. Cell. Biol. *7*, 4218–4224.
10. Koeffler, H. P. & Golde, D. W. (1978) Acute myelogenous leukemia: A human cell line responsive to colony-stimulating activity. Science *200*, 1153–1154.
11. Bradford, M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. *72*, 248–254.
12. Dünges, W. & Seiler, N. (1978) High performance liquid chromatographic separation of esters of 4-hydroxy-ethyl-7-methoxy-coumarin. A method for the determination of acidic compounds in the picomole range. J. Chromatogr. *145*, 483–488.
13. Plunkett, W., Iacoboni, S., Estey, E., Danhauser, L., Liliemark, J. O. & Keating, M. J. (1985) Pharmacologically directed ara-C therapy for refractory leukemia. Seminars in Oncology *12*, 20–30.
14. Estey, E. H., Keating, M. J., McCredie, K. B., Freireich, E. J. & Plunkett, W. (1990) Cellular ara-CTP pharmacokinetics, response, and caryotype in newly diagnosed acute myelogenous leukemia. Leukemia *4*, 95–99.
15. Liliemark, J. O. & Plunkett, W. (1986) Regulation of 1-β-D-arabinofuranosylcytosine 5′-triphosphate accumulation in human leukemia cells by deoxycytidine 5′-triphosphate. Cancer Research *46*, 1079–1083.

Dr. Peter Chiba
Department of Medical Chemistry
University of Vienna
Währingerstraße 10
A-1090 Vienna